OLIGONUCLEOTIDE PROBES FOR DETECTING *Enterobacteriaceae* AND QUINOLONE-RESISTANT *Enterobacteriaceae*

This invention was made in the Centers for Disease Control and Prevention, an agency of the United States Government. The U.S. Government has certain rights in this invention.

Technical Field of the Invention

This invention relates in general to the field of diagnostic microbiology. In particular, the invention relates to the species-specific detection of *Enterobacteriaceae*.

Background of the Invention

Enterobacteriaceae is a family of closely related, Gram-negative organisms associated with gastrointestinal diseases and a wide range of opportunistic infections. They are leading causes of bacteremia and urinary tract infections and are associated with wound infections, pneumonia, meningitis, and various gastrointestinal disorders. (Farmer, J. J., III. Enterobacteriaceae: Introduction and Identification. in Murray, P. R., et al., *Manual of Clinical Microbiology*, Washington, D.C., ASM Press, 6th (32): 438-449 (1998)). Many of these infections are life threatening and are often nosocomial (hospital-acquired) infections. (Schaberg et al., *The Am. J. Med.*, 91:72s-75s (1991) and CDC NNIS System Report *Am. J. Infect. Control.*, 24:380-388 (1996)).

Conventional methods for isolation and identification of these organisms include growth on selective and/or differential media followed

by biochemical tests of the isolated organism. Total incubation times require 24-48 hours. Slow-growing or fastidious strains require extended incubation times. An additional 18-24 hours is required for susceptibility testing, usually by disk diffusion or broth dilution. More recently, the identification of bacteria by direct hybridization of probes to bacterial genes or by detection of amplified genes has proven to be more time efficient.

Quinolones are broad-spectrum antibacterial agents effective in the treatment of a wide range of infections, particularly those caused by Gram-negative pathogens. (Stein, *Clin. Infect. Diseases,* 23(Suppl 1):S19-24 (1996) and Maxwell, *J. Antimicrob. Chemother.,* 30:409-416 (1992)). For example, nalidixic acid is a first-generation quinolone. Ciprofloxacin is an example of a second generation quinolone, which is also a fluoroquinolone. Sparfloxacin is an example of a third generation quinolone, which is also a fluoroquinolone. As used herein, the term "quinolone" is intended to include this entire spectrum of antibacterial agents, including the fluoroquinolones. This class of antibiotics has many advantages, including oral administration with therapeutic levels attained in most tissues and body fluids, and few drawbacks. As a result, indiscriminate use has led to the currently increasing incidence of quinolone/fluoroquinolone resistance. Hooper, *Adv. Expmtl. Medicine and Biology*, 390:49-57 (1995). Mechanisms of resistance to quinolones include alterations in DNA gyrase and/or topoisomerase IV and decreased intracellular accumulation of the antibiotic due to alterations in membrane proteins. (Hooper et al., *Antimicrob. Agents Chemother.,* 36:1151-1154 (1992)).

The primary target of quinolones, including the fluoroquinolones, in Gram-negative bacteria is DNA gyrase, a type II topoisomerase required for DNA replication and transcription. (Cambau et al., *Drugs,* 45(Suppl. 3):15-23 (1993) and Deguchi et al., *J. Antimicrob. Chemother.,* 40:543-549 (1997)). DNA gyrase, composed of two A subunits and two B subunits, is encoded by the *gyr*A and *gyr*B genes. Resistance to quinolones has been shown to be associated most frequently with alterations in *gyr*A. (Yoshida et al., *Antimicrob. Agents Chemother.* 34:1271-1272 (1990)). These mutations are localized at the 5' end of the gene (nucleotides 199-318 in the *E. coli* gene sequence) in an area designated as the quinolone resistance-determining region, or QRDR,

located near the active site of the enzyme, Tyr-122. (Hooper, *Adv. Expmtl. Medicine and Biology,* 390:49-57 (1995)).

Previous studies of fluoroquinolone-resistant strains of *Escherichia coli, Citrobacter freundii, Serratia marcescens* and *Enterobacter cloacae* have revealed that codons 81, 83, and 87 of *gyr*A are the sites most frequently mutated in Gram-negative organisms. (Nishino et al., *FEMS Microbiology Letters,* 154:409-414 (1997), and Kim et al., *Antimicrob. Agents Chemother.*, 42:190-193 (1998)). However, the association of *gyr*A mutations with fluoroquinolone resistance in *Enterobacter aerogenes, Klebsiella oxytoca,* and *Providencia stuartii* has not been established.

Previous publications have referred to the use of *gyr*A sequences to identify species within a single genus, such as Husmann et al., *J. Clin. Microbiol.,* 35(9):2398-2400 (1997) for Campylobacters, and Guillemin et al., *Antimicrob. Agents Chemo.,* 39(9):2145-2149 (1995) for Mycobacterium. The complete gene sequences of DNA gyrase A has previously been published for *Escherichia coli* (Swanberg, et al., *J. Mol. Biol.*, 197:729-736 (1987)) and *Serratia marcescens* (Kim et al., *Antimicrob. Agents Chemother.*, 42:190-193 (1998)). Fragments of *gyr*A including the QRDR have been published for *Enterobacter cloacae* (Deguchi, *J. Antimicrob. Chemother.* 40:543-549 (1997)) and *Citobacter freundii* (Nishino et al., *FEMS Microbiology Letters,* 154:409-414 (1997)). Additionally, the putative *gyr*A sequence for *Klebsiella pneumoniae* was published (Dimri et al., *Nucleic Acids Research,* 18:151-156 (1990)), however, the present invention demonstrates that the most likely organism used in that work was *Klebsiella oxytoca.*

The prior art has not provided enough information about different *Enterobacteriaceae* to develop probes capable of distinguishing between as many species as desirable, nor for determining the quinolone resistance-status of the species. It would be desirable to characterize additional *gyr*A genes and mutations from quinolone-resistant *Enterobacteriaceae* for species-specific identification and quinolone resistance determination using oligonucleotide probes.

Summary of the Invention

The present invention relates to oligonucleotide probes for detecting *Enterobacteriaceae* species. Unique *gyr*A coding regions permit the development of probes specific for identifying eight different species: *Escherichia coli*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Providencia stuartii* and *Serratia marcescens*. The invention thereby provides methods for the species-specific identification of these *Enterobacteriaceae* in a sample, and detection and diagnosis of *Enterobacteriaceae* infection in a subject.

Furthermore, the described unique DNA sequences from the 5' end of *gyr*A, within or flanking the quinolone resistance-determining region, permit the development of probes specific for determining the quinolone-resistant status of eight different species: *Escherichia coli*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Providencia stuartii* and *Serratia marcescens*. The invention thereby provides methods for the species-specific identification of these quinolone-resistant *Enterobacteriaceae*, and detection and diagnosis of quinolone-resistant *Enterobacteriaceae* infection in a subject.

Therefore, it is an object of the invention to provide improved materials and methods for detecting and differentiating *Enterobacteriaceae* species and/or quinolone resistance in the clinical laboratory and research settings.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

Brief Description of the Drawings

Figures 1A and 1B show the nucleic acid sequence (SEQ ID NOS:1-8) alignments for a portion of the *gyr*A gene in *Escherichia coli* (EC), *Citrobacter freundii* (CF), *Enterobacter aerogenes* (EA), *Enterobacter cloacae* (ECL), *Klebsiella oxytoca* (KO), *Klebsiella pneumoniae* (KP), *Providencia stuartii* (PS) and *Serratia marcescens* (SM).

Figure 2 shows the DNA sequence (SEQ ID NOS:9-16) similarity of the quinolone resistance-determining region (QRDR) in

Escherichia coli, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Providencia stuartii* and *Serratia marcescens*.

Figure 3 shows the deduced amino acid sequences (SEQ ID NOS:36-43) of the QRDR for *Escherichia coli*, *Citobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Providencia stuartii*, and *Serratia marcescens*.

Figures 4A and 4B show the alterations in GyrA amino acid sequences and susceptibilities of quinolone resistant clinical isolates of *Escherichia coli*, *Citobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Providencia stuartii*, and *Serratia marcescens*.

Detailed Description of the Invention

The present invention provides a simple, rapid and useful method for differentiating *Enterobacteriaceae* species and determining their quinolone-resistance status. This invention provides materials and methods to apply the species-specific probes to isolated DNA from host samples for an *in vitro* diagnosis of *Enterobacteriaceae* infection.

The present invention provides the nucleic acid sequences of conserved and unique regions of the *gyr*A gene of the following species of the Family *Enterobacteriaceae*: *Escherichia coli*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Providencia stuartii* and *Serratia marcescens*. The present invention provides the nucleic acid sequences of the quinolone resistance-determining region (QRDR) and surrounding regions of *gyr*A of each species listed above.

DNA sequence analyses revealed that *gyr*A is unique to each species and highly conserved within the species. However, the *gyr*A mutations resulting in amino acid substitutions which confer quinolone resistance vary in number, type, and position depending on the species. The invention demonstrates that these unique sequences can be used for identification of enteric organisms (genus and species) as well as detection of quinolone resistance within a given species. In addition, comparisons of *Enterobacteriaceae gyr*A with *gyr*A sequences from bacteria not closely

related to *Enterobacteriaceae* species suggest that *gyr*A sequences are unique for all bacterial species and may be used for identification of any species.

The invention provides unique, isolated nucleic acids containing regions of specificity for eight different members of the Family *Enterobacteriaceae*. These nucleic acids are from the *gyrA* gene of the *Enterobacteriaceae* genome. In particular, the invention provides isolated nucleic acids from *Escherichia coli* (SEQ ID NO:1), *Citrobacter freundii* (SEQ ID NO:2), *Enterobacter aerogenes* (SEQ ID NO:3), *Enterobacter cloacae* (SEQ ID NO:4), *Klebsiella oxytoca* (SEQ ID NO:5), *Klebsiella pneumoniae* (SEQ ID NO:6), *Providencia stuartii* (SEQ ID NO:7) and *Serratia marcescens* (SEQ ID NO:8). These sequences can be used to identify and distinguish the respective species of *Enterobacteriaceae*. Figures 1A and 1B show the nucleic acids of SEQ ID NOS:1-8. The sequences correspond to nucleotides #25-613, based on the *E. coli gyr*A sequence numbers of Swanberg et al., *J. Mol. Biol.*, 197:729-736 (1987).

The invention also provides unique, isolated nucleic acids from the quinolone resistance-determining region of *Escherichia coli* (SEQ ID NO:9), *Citrobacter freundii* (SEQ ID NO:10), *Enterobacter aerogenes* (SEQ ID NO:11), *Enterobacter cloacae* (SEQ ID NO:12), *Klebsiella oxytoca* (SEQ ID NO:13), *Klebsiella pneumoniae* (SEQ ID NO:14), *Providencia stuartii* (SEQ ID NO:15) and *Serratia marcescens* (SEQ ID NO:16). These sequences can be used to determine the quinolone resistance status of each species. The QRDR nucleic acids are shown in Figure 2.

Furthermore, the invention provides specific examples of isolated nucleic acid probes derived from the above nucleic acid sequences which may be used as species-specific identifiers of *Escherichia coli* (SEQ ID NO:17), *Citrobacter freundii* (SEQ ID NO:18), *Enterobacter aerogenes* (SEQ ID NO:19), *Enterobacter cloacae* (SEQ ID NO:20), *Klebsiella oxytoca* (SEQ ID NO:21), *Klebsiella pneumoniae* (SEQ ID NO:22), *Providencia stuartii* (SEQ ID NO:23) and *Serratia marcescens* (SEQ ID NO:24).

The invention also provides specific examples of isolated nucleic acid probes derived from the QRDR of the above nucleic acid sequences which may be used as determinants of quinolone resistance for *Escherichia coli* (SEQ ID NOS:25 and 26), *Citrobacter freundii* (SEQ ID

NO:27), *Enterobacter aerogenes* (SEQ ID NO:28), *Enterobacter cloacae* (SEQ ID NO:29), *Klebsiella oxytoca* (SEQ ID NO:30), *Klebsiella pneumoniae* (SEQ ID NO:31), *Providencia stuartii* (SEQ ID NO:32) and *Serratia marcescens* (SEQ ID NO:33).

Such probes can be used to selectively hybridize with samples containing nucleic acids from species of *Enterobacteriaceae*. The probes can be incorporated into hybridization assays using polymerase chain reaction, ligase chain reaction, or oligonucleotide arrays on chips or membranes, for example. Additional probes can routinely be derived from the sequences given in SEQ ID NOs:1-8, which are specific for identifying the respective species or for determining quinolone resistance. Therefore, the probes shown in SEQ ID NOs:17-24 and 25-33 are only provided as examples of the species-specific probes or quinolone resistance-determining probes, respectively, that can be derived from SEQ ID NOs:1-8.

By "isolated" is meant nucleic acid free from at least some of the components with which it naturally occurs. By "selective" or "selectively" is meant a sequence that does not hybridize with other nucleic acids to prevent adequate determination of an *Enterobacteriaceae* species or quinolone resistance, depending upon the intended result. As used herein to describe nucleic acids, the term "selectively hybridizes" excludes the occasional randomly hybridizing nucleic acids, and thus has the same meaning as "specifically hybridizing".

A hybridizing nucleic acid should have at least 70% complementarity with the segment of the nucleic acid to which it hybridizes. The selectively hybridizing nucleic acids of the invention can have at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, and 99% complementarity with the segment of the sequence to which it hybridizes. The exemplary probes shown in SEQ ID NOs:17-24 and 25-33 are designed to have 100% hybridization with the target DNA.

The invention contemplates sequences, probes and primers which selectively hybridize to the complementary, or opposite, strand of nucleic acid as those specifically provided herein. Specific hybridization with nucleic acid can occur with minor modifications or substitutions in the nucleic acid, so long as functional species-specific or quinolone resistance determining hybridization capability is maintained. By "probe" is meant a nucleic acid sequence that can be used as a probe or primer for selective

hybridization with complementary nucleic acid sequences for their detection or amplification, which probe can vary in length from about 5 to 100 nucleotides, or preferably from about 10 to 50 nucleotides, or most preferably about 25 nucleotides. The invention provides isolated nucleic acids that selectively hybridize with the species-specific nucleic acids under stringent conditions. *See generally,* Maniatis, et al., Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1982) latest edition.

Molecular biology techniques permit the rapid detection of hybridization, such as through confocal laser microscopy and high density oligonucleotide arrays and chips. See, Kozal et al., *Nat. Med.,* 2(7): 753-759 (1996), Schummer et al., *Biotech.,* 23:1087-1092 (1997) or Lockhart et al., *Nat. Biotech.* 14:1675-1680 (1996). Another example of a detection format is the use of controlled electric fields that permit the rapid determination of single base mismatches, as described in Sosnowski et al., *Proc. Natl. Acad. Sci. USA,* 94:1119-1123 (1997). The invention contemplates the use of the disclosed nucleic acid sequences and probes derived therefrom with these currently available techniques and those new techniques discovered in the future.

If used as primers, the invention provides compositions including at least two oligonucleotides (i.e., nucleic acids) that hybridize with different regions of DNA so as to amplify the desired region between the two primers. Depending on the length of the probe or primer, the target region can range between 70% complementary bases and full complementarity and still hybridize under stringent conditions. For example, for the purpose of diagnosing the presence of the *Enterobacteriaceae* in a clinical sample, the degree of complementarity between the nucleic acid (probe or primer) and the target sequence to which it hybridizes (e.g., *Enterobacteriaceae* DNA from a sample) is at least enough to distinguish hybridization with a non-target nucleic acid from other *Enterobacteriaceae*. The invention provides examples of nucleic acids having sequences unique to *Enterobacteriaceae* such that the degree of complementarity required to distinguish selectively hybridizing from nonselectively hybridizing nucleic acids under stringent conditions can be clearly determined for each nucleic acid.

Alternatively, the nucleic acid probes can be designed to have homology with nucleotide sequences present in more than one species of

Enterobacteriaceae. Such a nucleic acid probe can be used to selectively identify a group of *Enterobacteriaceae* species. Additionally, the invention provides that the nucleic acids can be used to differentiate *Enterobacteriaceae* species in general from other species. Such a determination is clinically significant, since therapies for these infections differ.

The invention further provides methods of using the nucleic acids to detect and identify the presence of *Enterobacteriaceae*, or particular species thereof. The methods involve the steps of obtaining a sample suspected of containing *Enterobacteriaceae*. The sample, such as blood, urine, lung lavage fluids, spinal fluid, bone marrow aspiration, vaginal mucosa, tissues, etc., may be taken from an individual, or taken from the environment. The *Enterobacteriaceae* cells in the sample can then be lysed, and the DNA released (or made accessible) for hybridization with oligonucleotide probes.

The DNA sample is preferably amplified prior to hybridization using primers derived from the *gyr*A regions of the *Enterobacteriaceae* DNA that are designed to amplify several species. Examples of such primers are shown below as GYRA6 (SEQ ID NO:34) and or GYRA631R (SEQ ID NO:35). Detection of and/or the determination of quinolone resistance in the target species of *Enterobacteriaceae* is achieved by hybridizing the amplified *gyr*A DNA with an *Enterobacteriaceae* species-specific probe that selectively hybridizes with the DNA. Detection of hybridization is indicative of the presence of the particular species of *Enterobacteriaceae* or quinolone resistance, depending upon the probe. In the case where the species of *Enterobacteriaceae* is known, for example through previous hybridization with a species-specific identifying probe of SEQ ID NOS:17-24, the lack of subsequent hybridization with a species-specific quinolone resistance-determining probe of SEQ ID NOS:25-33 is indicative of quinolone resistance in the sample.

Preferably, detection of nucleic acid hybridization can be facilitated by the use of reporter or detection moieties. For example, the species-specific probes can be labeled with digoxigenin, and a universal-*Enterobacteriaceae* species probe can be labeled with biotin and used in a streptavidin-coated microtiter plate assay. Other examples of detectable

moieties include radioactive labeling, enzyme labeling, and fluorescent labeling.

The invention further contemplates a kit containing one or more species-specific and/or quinolone resistance-determining probes, which can be used for the identification and/or quinolone resistance determination of particular *Enterobacteriaceae* species. Such a kit can also contain the appropriate reagents for hybridizing the probe to the sample and detecting bound probe. The invention may be further demonstrated by the following non-limiting examples.

Examples

Example 1

In this Example, the DNA sequence of the *gyr*A was determined for eight species of*Enterobacteriaceae*. Oligonucleotide primers were designed from conserved *gyr*A gene sequences flanking the QRDR and used to amplify and sequence the 5' region of *gyr*A from ATCC type strains and fluoroquinolone-resistant clinical isolates. The nucleotide and the inferred amino acid sequences were aligned and compared.

The QRDR sequences from 60 clinical isolates with decreased fluoroquinolone susceptibilities were analyzed for alterations associated with fluoroquinolone resistance. The primer sequences at the 3' and 5' ends have been removed leaving nucleotides #25-613, based on the *E. coli gyr*A sequence numbers of Swanberg et al., *J. Mol. Biol.*, 197:729-736 (1987). The organisms, abbreviations and ATCC type strain designation numbers are as follows.

EC = *Escherichia coli* (*E. coli*) ATCC 11775
CF = *Citrobacter freundii* (*C. freundii*) ATCC 8090
EA = *Enterobacter aerogenes* (*E. aerogenes*) ATCC 13048
ECL = *Enterobacter cloacae* (*E. cloacae*) ATCC 13047
KO = *Klebsiella oxytoca* (*K. oxytoca*) ATCC 13182
KP = *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 13883
PS = *Providencia stuartii* (*P. stuartii*) ATCC 29914
SM = *Serratia marcescens* (*S. marcescens*) ATCC 13880

Amplification of *gyrA*

Bacterial strains and determination of antibiotic susceptibility profiles.

Type strains of *Enterobacteriaceae* were from American Type Culture Collection (ATCC). Fluoroquinolone resistant and susceptible clinical isolates were selected from the Intensive Care Antimicrobial Resistance Epidemiology (ICARE) study, collected from 39 hospitals across the U.S. between June, 1994 and April 1997 (Archibald et al., *CID*, 24(2):211-215 (1997)). ICARE isolates were screened to exclude duplicate strains from the same patient.

Minimal inhibitory concentrations (MICs) were determined by the broth microdilution method with cation-adjusted Müeller-Hinton broth according to the methods of the National Committee for Clinical Laboratory Standards (NCCLS M7-A4 (1997)). Ciprofloxacin was purchased from Bayer Corporation (West Haven, CT), ofloxacin and nalidixic acid were from Sigma (St. Louis, MO) and sparfloxacin was from Rhône-Poulenc Rorer (Collegeville, PA).

Amplification of 5' region of *gyrA*.

Oligonucleotide primers were designed based on homologous regions of *gyrA* sequences in *E. coli* (Swanberg et al., *J. Mol. Biol.*, 1987. 197:729-736) and *K. oxytoca* (published by Dimri et al., *Nuc. Acids Res.*, 1990. 18:(1):151-156 as *K. pneumonia*), as follows:

GYRA6
5'-CGACCTTGCGAGAGAAAT-3' (SEQ ID NO:34)

GYRA631R
5'-GTTCCATCAGCCCTTCAA-3' (SEQ ID NO:35)

Primer GYRA6 corresponds to nucleotides 6 to 23 and primer GYRA631R is complementary to nucleotides 610 to 631 of the *E. coli gyrA* sequence.

DNA fragments were amplified from chromosomal DNA in cell lysates. Amplifications were carried out in a GeneAmp 9600 PCR System (Perkin-Elmer, Applied Biosystems Division, Foster City, CA) in 50 µl volume containing 50 pmol of each primer, 200 µM deoxynucleoside triphosphates, 10 ul cell lysate containing approximately 100 ng template

DNA, 1X reaction buffer with 1.5 mM $MgCl_2$ and 1 U native Taq polymerase (Perkin Elmer). An initial 4 minute period of denaturation at 94°C was followed by 30 cycles including: denaturation for 1 minute at 94°C, annealing for 30 seconds at 55°C, extending for 45 seconds at 72°C, followed by a final cycle of 72°C for 10 minutes. Amplification products were visualized by agarose gel electrophoresis and ethidium bromide staining to determine specificity and size of gene fragments. PCR products were purified on QIAquick spin columns (QIAGEN, Chatsworth, CA) and sequenced with the ABI Prism Dye Terminator or dRhodomine Terminator Cycle Sequencing Kit and an ABI 377 automated sequencer (Perkin Elmer). To eliminate errors due to amplification artifacts, the forward and reverse sequences of each QRDR were determined using products from independent PCR reactions. The GCG (Genetics Computer Group, Madison, WI) analyses programs were used for the construction of DNA and amino acid sequence alignments.

The resultant sequences of the *gyrA* regions for *Escherichia coli*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Providencia stuartii* and *Serratia marcescens* are shown below in Table 1 and in Figures 1A-1B. The sequences provided correspond to nucleotide positions 25 to 613 of the *gyrA* regions for *Escherichia coli*.

TABLE 1

Gyrase A 5' Region Sequences

Escherichia coli

ACACCGGT CAACATTGAG GAAGAGCTGA AGAGCTCCTA TCTGGATTAT
GCGATGTCGG TCATTGTTGG CCGTGCGCTG CCAGATGTCC GAGATGGCCT
GAAGCCGGTA CACCGTCGCG TACTTTACGC CATGAACGTA CTAGGCAATG
ACTGGAACAA AGCCTATAAA AAATCTGCCC GTGTCGTTGG TGACGTAATC
GGTAAATACC ATCCCCATGG TGACTCGGCG GTTTATGACA CGATCGTCCG
TATGGCGCAG CCATTCTCGC TGCGTTACAT GCTGGTAGAC GGTCAGGGTA
ACTTCGGTTC CATCGACGGC GACTCTGCGG CGGCAATGCG TTATACGGAA
ATCCGTCTGG CGAAAATTGC CCATGAACTG ATGGCTGATC TCGAAAAAGA
GACGGTCGAT TTCGTTGATA ACTATGACGG TACGGAAAAA ATTCCGGACG
TCATGCCAAC CAAAATTCCT AACCTGCTGG TGAACGGTTC TTCCGGTATC

GCCGTAGGTA TGGCAACCAA CATCCCGCCG CACAACCTGA CGGAAGTCAT CAACGGTTGT CTGGCGTATA TCGATGATGA AGACATCAGC A (SEQ ID NO:1)

Citrobacter freundii

ACACCGGT CAACATTGAG GAAGAGCTGA AGAGCTCCTA TCTGGATTAT GCGATGTCGG TCATTGTTGG CCGTGCGCTG CCAGACGTCC GAGATGGCCT GAAGCCGGTT CACCGTCGCG TACTTTACGC CATGAACGTA TTGGGCAACG ACTGGAATAA AGCCTATAAA AAATCTGCCC GTGTCGTTGG TGACGTAATC GGTAAATACC ACCCTCATGG TGATACCGCC GTTTACGACA CCATTGTTCG TATGGCGCAG CCATTCTCCT TGCGTTACAT GCTGGTAGAT GGTCAGGGTA ACTTTGGTTC TGTCGATGGC GACTCCGCAG CGGCGATGCG TTATACGGAA ATCCGTATGT CGAAAATCGC CCATGAGCTG ATGGCTGACC TGGAAAAAGA AACGGTTGAT TTCGTCGATA ACTACGACGG CACCGAACAA ATTCCTGACG TCATGCCGAC CAAAATTCCT AACCTGCTGG TGAACGGTTC GTCCGGTATC GCGGTAGGTA TGGCGACCAA CATTCCGCCG CACAACCTGA CTGAAGTGAT CAACGGCTGT CTGGCATATA TTGACGATGA AGACATCAGC A (SEQ ID NO:2)

Enterobacter aerogenes

ACACGGGT CAACATTGAG GAAGAGCTGA AAAGCTCGTA TCTGGATTAT GCGATGTCGG TCATTGTTGG CCGTGCGCTG CCGGATGTCC GAGATGGCCT GAAGCCGGTA CACCGTCGCG TACTATACGC CATGAACGTA TTGGGCAATG ACTGGAACAA AGCCTATAAA AAATCAGCCC GTGTCGTTGG CGACGTAATC GGTAAATACC ACCCGCATGG TGATACCGCC GTTTATGACA CCATCGTACG TATGGCGCAG CCGTTCTCCT TGCGTTATAT GCTGGTCGAT GGCCAGGGTA ACTTTGGTTC TGTCGATGGC GACTCCGCTG CAGCGATGCG TTATACGGAA ATCCGTATGT CGAAGATCGC TCATGAGCTG ATGGCCGATC TCGAAAAAGA GACGGTTGAT TTCGTCGACA ACTATGACGG CACGGAGAAA ATCCCTGACG TCATGCCGAC AAAAATCCCT AACCTGCTGG TGAACGGTTC TTCCGGTATC GCCGTAGGTA TGGCGACCAA CATTCCGCCG CATAACCTGA CGGAAGTTAT CAACGGCTGC CTGGCATACG TTGATAACGA AGACATCAGC A (SEQ ID NO:3)

Enterobacter cloacae

ACACCGGTTA ACATCGAGGA AGAGCTGAAG AGCTCCTATC TGGACTATGC GATGTCGGTC ATTGTTGGCC GTGCGCTGCC GGACGTCCGC GATGGCCTGA AGCCGGTACA CCGTCGCGTA CTATACGCCA TGAACGTATT GGGCAATGAC

TGGAATAAAG CCTACAAAAA ATCTGCCCGT GTCGTTGGTG ACGTAATCGG TAAATACCAT CCCCATGGTG ATTCCGCGGT GTACGACACC ATCGTTCGTA TGGCGCAGCC TTTCTCGCTG CGTTACATGC TGGTAGATGG TCAGGGTAAC TTTGGTTCTA TCGACGGCGA CTCCGCCGCG GCAATGCGTT ATACGGAAAT CCGTCTGGCG AAAATTGCCC ATGAGCTGAT GGCCGACCTG GAAAAAGAGA CGGTTGATTT CGTTGATAAC TACGATGGCA CGGAAAAAAT TCCTGACGTC ATGCCAACGA AGATCCCTAA CCTGCTGGTG AACGGTTCGT CCGGTATCGC CGTAGGGATG GCGACCAACA TTCCGCCGCA CAACATCACC GAAGTGATCA ACGGCTGCCT GGCCTATATC GACGATGAAG ACATCAGCA (SEQ ID NO:4)

Klebsiella oxytoca

ACACCGGT CAACATTGAG GAAGAGCTGA AGAGCTCCTA TCTGGATTAT GCGATGTCGG TCATTGTTGG CCGTGCGCTG CCGGATGTCC GAGATGGCCT GAAGCCGGTA CACCGTCGCG TACTATACGC CATGAACGTA TTGGGCAATG ACTGGAACAA AGCCTATAAA AAATCTGCCC GTGTCGTGGG TGACGTCATC GGTAAATACC ACCCTCATGG TGATACTGCC GTATACGACA CCATTGTACG TATGGCGCAG CCATTCTCCC TGCGTTACAT GCTGGTAGAT GGCCAGGGTA ACTTTGGTTC GGTCGACGGC GACTCCGCCG CAGCGATGCG TTATACGGAA ATCCGTATGT CGAAGATCGC CCATGAACTG ATGGCCGACC TCGAAAAAGA GACGGTGGAT TTCGTCGATA ACTATGACGG CACGGAGAAA ATCCCTGACG TTATGCCGAC CAAAATCCCG AACCTGCTAG TCAACGGTTC GTCCGGTATC GCGGTAGGTA TGGCGACTAA TATTCCGCCG CACAACCTGA CCGAAGTGAT CAACGGCTGT CTGGCCTACG TTGAAAACGA AGACATCAGC A (SEQ ID NO:5)

Klebsiella pneumoniae

ACACCGGT CAACATTGAG GAAGAGCTTA AGAACTCTTA TCTGGATTAT GCGATGTCGG TCATTGTTGG CCGTGCGCTG CCGGATGTCC GAGATGGCCT GAAGCCGGTA CACCGTCGCG TACTTTACGC CATGAACGTA TTGGGCAATG ACTGGAACAA AGCCTATAAA AAATCAGCCC GTGTCGTTGG TGACGTAATC GGTAAATACC ACCCGCACGG CGACTCCGCG GTATACGACA CCATCGTGCG TATGGCGCAG CCGTTCTCGC TGCGTTACAT GCTGGTGGAC GGCCAGGGTA ACTTTGGTTC CATCGACGGC GACTCCGCCG CGGCGATGCG TTATACCGAA ATTCGTCTGG CGAAAATCGC TCATGAGCTG ATGGCCGATC TTGAAAAAGA GACGGTCGAT TTCGTCGACA ACTATGACGG TACGGAGCGT ATTCCGGACG TCATGCCGAC CAAAATTCCT AACCTGCTGG TGAACGGCGC CTCCGGGATC GCCGTAGGGA TGGCCACCAA CATACCGCCA CATAACCTGA CGGAAGTGAT

TAACGGCTGT CTGGCGTATG TTGACGATGA AGACATCAGC A (SEQ ID NO:6)

Providencia stuartii

ACACCGGT CAATATCGAA GAAGAACTCA AAAGTTCGTA TTTGGATTAT GCGATGTCCG TTATTGTCGG GCGCGCGCTT CCAGATGTTC GAGATGGACT GAAGCCAGTACACCGCAGAG TACTGTTTGC GATGAATGTA TTGGGAAATG ATTGGAATAA ACCCTATAAA AAATCTGCCC GTATAGTCGG GGACGTTATC GGTAAATACC ATCCACATGG TGATAGCGCT GTTTATGAGA CAATCGTTCG TCTTGCTCAG CCTTTTTCTA TGCGTTATAT GCTGGTAGAT GGTCAGGGGA ACTTTGGTTC AGTTGACGGA GATTCCGCAG CTGCAATGCG TTATACGGAA ATCCGTATGG CGAAAATTGC CCATGAAATG TTAGCGGATC TTGAAAAAGA GACCGTTGAT TTCGTCCCAA ACTATGATGG TACAGAGCAA ATCCCTGAAG TTATGCCTAC GAAAATCCCT AACCTATTGG TTAATGGTTC GTCAGGTATT GCTGTTGGGA TGGCAACGAA CATTCCTCCA CACAACCTAG GGGAAGTGAT CAGCGGTTGC CTTGCTTATA TAGATGATGA AGATATTAGC A (SEQ ID NO:7)

Serratia marcescens

ACACCGGT AAACATCGAA GACGAGTTGA AAAACTCGTA TCTGGACTAT GCGATGTCCG TTATTGTCGG ACGTGCCCTG CCAGATGTTC GTGATGGACT GAAGCCGGTT CACCGCCGCG TTCTGTACGC GATGAGCGTA TTGGGTAACG ACTGGAATAA ACCATACAAG AAATCGGCCC GTGTCGTCGG GGACGTGATC GGTAAATATC ACCCGCACGG TGACAGCGCG GTTTACGACA CTATCGTGCG TATGGCTCAG CCGTTTTCAC TGCGCTACAT GCTGGTGGAC GGTCAGGGTA ACTTCGGTTC CGTCGACGGC GACTCCGCGG CGGCGATGCG TTATACCGAA GTGCGCATGT CCAAGATTGC TCACGAACTG TTGGCGGATC TGGAAAAAGA AACCGTCGAC TTCGTGCCTA ACTATGATGG CACCGAGCAG ATCCCGGCCG TCATGCCGAC CAAGATCCCG AACCTGCTGG TCAACGGCTC GTCGGGCATC GCCGTGGGCA TGGCTACCAA TATTCCGCCG CACAACCTGG CGGAAGTCGT CAACGGCTGC CTGGCCTATA TCGACGATGA AAACATCAGC A (SEQ ID NO:8)

The QRDR sequences from positions 199 to 318 (relative to *E. coli*) are shown below in Table 2.

TABLE 2

Quinolone Resistance-Determining Region Sequences

Escherichia coli

GCCCG TGTCGTTGGT GACGTAATCG GTAAATACCA TCCCCATGGT GACTCGGCGG TTTATGACAC GATCGTCCGT ATGGCGCAGC CATTCTCGCT GCGTTACATG CTGGTAGACG GTCAG (SEQ ID NO:9)

Citrobacter freundii

GCCCG TGTCGTTGGT GACGTAATCG GTAAATACCA CCCTCATGGT GATACCGCCG TTTACGACAC CATTGTTCGT ATGGCGCAGC CATTCTCCTT GCGTTACATG CTGGTAGATG GTCAG (SEQ ID NO:10)

Enterobacter aerogenes

GC CCGTGTCGTT GGCGACGTAA TCGGTAAATA CCACCCGCAT GGTGATACCG CCGTTTATGA CACCATCGTA CGTATGGCGC AGCCGTTCTC CTTGCGTTAT ATGCTGGTCG ATGGCCAG (SEQ ID NO:11)

Enterobacter cloacae

GC CCGTGTCGTT GGTGACGTAA TCGGTAAATA CCATCCCCAT GGTGATTCCG CGGTGTACGA CACCATCGTT CGTATGGCGC AGCCTTTCTC GCTGCGTTAC ATGCTGGTAG ATGGTCAG (SEQ ID NO:12)

Klebsiella oxytoca

GCCCGTGTC GTGGGTGACG TCATCGGTAA ATACCACCCT CATGGTGATA CTGCCGTATA CGACACCATT GTACGTATGG CGCAGCCATT CTCCCTGCGT TACATGCTGG TAGATGGCCA G (SEQ ID NO:13)

Klebsiella pneumoniae

GC CCGTGTCGTT GGTGACGTAA TCGGTAAATA CCACCCGCAC GGCGACTCCG CGGTATACGA CACCATCGTG CGTATGGCGC AGCCGTTCTC GCTGCGTTAC ATGCTGGTGG ACGGCCAG (SEQ ID NO:14)

Providencia stuartii

GCCCGTATAG TCGGGGACGT TATCGGTAAA TACCATCCAC ATGGTGATAG CGCTGTTTAT GAGACAATCG TTCGTCTTGC TCAGCCTTTT TCTATGCGTT ATATGCTGGT AGATGGTCAG (SEQ ID NO:15)

Serratia marcescens
GCCCGTGTC GTCGGGGACG TGATCGGTAA ATATCACCCG CACGGTGACA GCGCGGTTTA CGACACTATC GTGCGTATGG CTCAGCCGTT TTCACTGCGC TACATGCTGG TGGACGGTCA G (SEQ ID NO:16)

Oligonucleotide primers GYRA6 and GYRA631R successfully amplified the expected 626 bp DNA fragment from *Escherichia coli*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Providencia stuartii* and *Serratia marcescens* (**Figs. 1A-1B**). In additional experiments, amplification with GYRA6 and GYRA631 produced the expected *GYR*A fragment from *S. typhimurium* (data not shown).

The PCR products were sequenced and the 120 bp regions of *gyr*A known as the QRDR were analyzed. Alignment of the QRDR DNA sequences of the type strains revealed numerous nucleotide substitutions when compared with the *E. coli* sequence (**Fig. 2**). Eighty-seven of 120 nucleotides (72.5%) were conserved. Similarity to the *E. coli* sequence varied from 93.3% for *E. cloacae* to 80.8% for *P. stuartii* (**Figs. 4A-4B**). Significant diversity was noted when the *gyr*A QRDR sequences of two species from one genus were aligned. *E. aerogenes* and *E. cloacae* shared 90.5% identity and*K. pneumoniae* and *K. oxytoca* shared 89.3 % identity in this region, less similarity than between several of the different genera.

The *gyr*A QRDR sequence of the *E. coli* type strain (ATCC 11775) was compared with the *E. coli* K12 *gyr*A sequence published by Swanberg and Wang (*J. Mol. Biol.* 197:729-736 (1997)) and 4 nucleotide differences were detected at positions 255 (C -> T), 267 (T -> C), 273 (C -> T), and 300 (T -> C).

When the QRDR sequence from the*K. pneumoniae* type strain was compared with the*gyr*A gene sequence from *K. pneumoniae* strain M5a1 published by Dimri and Das (*Nucleic Acids Research*, 18:151-156 (1990)), differences were detected in 15 of 120 nucleotides. Of these 15 nucleotides, only one resulted in an amino acid change. At nucleotide position 247 a T to A change altered the deduced amino acid from Ser-83 (ATCC type strain) to Thr (M5a1). When the M5a1 *gyr*A sequence was compared with that of the*K. oxytoca* type strain, only 4 nucleotide differences were detected. In addition, Ser was consistently found at position 83 in the fluoroquinolone-susceptible strains of *K. pneumoniae*

and Thr was consistently found at this position in the *K. oxytoca* strains (**Figs. 4A and 4B**). These data indicate that the Dimri and Das-sequence of the M5a1 strain most likely was from a strain of *K. oxytoca* and not *K. pneumoniae*.

In the sequence from the *S. marcescens* type strain (ATCC 13880), the QRDR was identical to the sequence published by Kim et al. (ATCC 14756)(*Antimicrob. Agents Chemother.*, 42:190-193 (1998)). One nucleotide difference was found in the flanking region (nt 321, T to C) with no change in amino acid sequence (data not shown). The *C. freundii* QRDR sequence was identical to that of Nishino et al. (*FEMS Microbiology Letters*, 154:409-414 (1997)), however, an additional 393 nucleotides are presented herein.

The deduced amino acid sequences of the QRDR were highly conserved (**Fig. 3**). *E. cloacae, K. pneumoniae* and *S. marcescens* shared identical amino acid sequences with *E. coli*. In *C. freundii, E. aerogenes* and *K. oxytoca*, one conservative substitution, Ser-83 to Thr was found. Only *P. stuartii* exhibited more than one amino acid substitution in this region. In this organism two conservative changes were detected, Val-69 to Ile and Asp-87 to Glu. In addition, the Leu-92 and Met-98 positions were reversed when compared with the amino acid sequences of other members of the *Enterobacteriaceae* family included in this study. The Glu at position 87 is typical for *gyr*A in Gram-positive organisms (Tankovic et al., *Antimicrob. Agents Chemother.*, 40:2505-2510 (1996)), but has not previously been described for a Gram-negative organism.

After determining the DNA sequence of the QRDR from the quinolone-susceptible type strains, the 5' region of *gyr*A in ciprofloxacin-resistant and -susceptible clinical isolates was amplified, sequenced, and analyzed for mutations leading to amino acid changes associated with fluoroquinolone resistance (**Figs. 4A and 4B**). Comparisons of the fluoroquinolone-susceptible type strain and the resistant clinical isolates of *E. coli* revealed single mutations in codon 83 in *gyr*A associated with low levels of resistance and double mutations (codons 83 and 87) with high levels of resistance ($\geq$16 ug/ml ciprofloxacin) as previously described (Vila et al., *Antimicrob. Agents Chemother.*, 38:2477-2479 (1994) and Heisig et al., *Antimicrob. Agents Chemother.*, 37:696-701 (1993)). However, in all other species in this study, high levels of resistance were found in strains with single as well as double *gyr*A

mutations. MICs varied significantly among strains with the same mutation, confirming that factors other than *gyr*A are involved in determining the level of resistance to fluoroquinolones (Everett et al., *Antimicrob. Agents Chemother.*, 40:2380-2386 (1996) and Piddock, *Drugs*, 49 (Suppl):29-35 (1995)).

All clinical isolates of *C. freundii* with reduced susceptibility to fluoroquinolones were found to have Thr-83 to Ile mutations, resulting from C-to-T substitutions at nucleotide position 248. Two isolates also displayed alterations of Asp-87 to Gly. However, as noted for isolate *C. freundii* 9023 (**Figs. 4A and 4B**), the presence of a double mutation was not required for high-level resistance (MICs of 16 μg/ml ciprofloxacin). The nucleotide substitutions in codon 83 of *E. aerogenes gyr*A (Thr-83 to Ile) were identical to those of *C. freundii*. No double mutations were detected in *gyr*A from 7 strains of *E. aerogenes* with reduced levels of susceptibility to fluoroquinolones. However, MICs of isolates with the single mutation ranged from 2 - 16 μg/ml ciprofloxacin.

Clinical isolates of *E. cloacae* exhibited numerous substitutions resulting in Ser-83 changes to Phe, Tyr, or Ile with no single amino acid change associated with either low level or high level resistance. There was no alteration of Ser-83 in the clinical isolate *E. cloacae* 1524 which had a marginal decrease in susceptibility to the fluoroquinolones. However, Asp-87 was changed to Asn. This alteration, found as part of a double mutation in *E. cloacae* 1224, may contribute to high-level resistance if additional changes occur in the QRDR of *E. cloacae* 1524.

K. pneumoniae isolates exhibited either single or double mutations involving Ser-83 and Asp-87, and ciprofloxacin MICs ranged from 1 - 16 μg/ml. Again, double mutations were not required for high-level resistance and no specific mutation (Ser-83 to Phe or Tyr) was associated with low or high levels of fluoroquinolone resistance.

K. oxytoca mutations were confined to the Thr-83 codon and were consistent C-to-T substitutions in the second position resulting in amino acid change to Ile, similar to *C. freundii* and *E. aerogenes*. MICs associated with this alteration ranged from 0.5 - 16 μg/ml ciprofloxacin.

Changes in the QRDR of *P. stuartii gyr*A were also confined to codon 83, however, the nucleotide substitutions varied. The single nucleotide substitutions included A-to-C at the first position or C-to- G at the third position, both resulting in Ser-to-Arg mutations, or G-to-T in the

second position resulting in Ser-to-Ile mutations. MICs ranged from 2 to 16 µg/ml ciprofloxacin.

S. marcescens displayed the greatest diversity in mutations with Gly-81, Ser-83, or Asp-87 involved. No double mutations were detected in the QRDR of gyrA from 6 fluoroquinolone-resistant clinical isolates. An unusual mutation of Gly-81 to Cys was found in two isolates. However, this mutation has been described in *E. coli* (Yoshida et al., *Antimicrob. Agents Chemother.*, 34:1271-1272 (1990)).

The data in this Example provides for the first time enough comparative nucleic acid sequence data for the *gyr*A gene to enable one to prepare probes that will selectively hybridize to target nucleic acid to identify the species and/or quinolone resistance of *Escherichia coli*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Providencia stuartii* and *Serratia marcescens*.

Example 2

Development of Probes

Identification of *Enterobacteriaceae* Species

Oligonucleotide probes can be selected for species-specific identification of *Enterobacteriaceae* in or near the QRDR of *gyr*A. The region which includes the codons most often associated with fluoroquinolone resistance (nucleotides 239-263) was not used for the reason that if identification were based on one or more nucleotide changes, the changes associated with resistance would interfere with identification. Each probe for identification was selected for maximum difference, and it is recognized that a smaller region within some probes could be used, based on single base changes. However, most of the probes have at least two nucleotide differences compared with the same region in other strains. When there were variations, other than those associated with resistance, within the susceptible and/or the resistance strains for any given species, the position of the probe was shifted to a region which was completely conserved for all strains sequenced. For this reason, the probes were in the region 5' of the QRDR.

TABLE 3

Oligonucleotide probes for identification of *Enterobacteriaceae*

E. coli 5' ACT TTA CGC CAT GAA CGT ACT AGG C 3' (SEQ ID NO:17) (144-168)

C. freundii 5' TGG GCA ACG ACT GGA ATA AAG CC 3' (SEQ ID NO:18) (164-186)

E. aerogenes 5' TTA TAT GCT GGT CGA TGG CCA G 3' (SEQ ID NO:19) (297-323)

E. cloacae 5' GCC GGA CGT CCG CGA TGG CCT 3' (SEQ ID NO:20) (102-122)

K. oxytoca 5' GTA GAT GGC CAG GGT AAC TTT GGT TCG GTC 3' (SEQ ID NO:21) (307-336)

K. pneumoniae 5' GTG CGT ATG GCG CAG CCG TTC TCG CTG 3' (SEQ ID NO:22) (268-294)

P. stuartii 5' CGT CTT GCT CAG CCT TTT TCT ATG C 3' (SEQ ID NO:23) (271-295)

S. marcescens 5' GGA ATA AAC CAT ACA AGA AA 3' (SEQ ID NO:24) (176-195)

Note: Numbers in parentheses refer to base positions in *E. coli* sequence

Fluoroquinolone resistance probes

Simultaneous identification of the species and mutations leading to resistance can be determined by using one of the above oligonucleotide probes in combination with the resistance probes set forth below. All oligonucleotide probes shown in Table 4 for quinolone resistance span the region containing the amino acid codons most frequently associated with resistance (nucleotides 239-263). Susceptible strains will hybridize to the resistance probe for that species and resistance

will be detected as one or more basepair mismatch with the susceptible strain sequence.

TABLE 4

Oligonucleotide probes for quinolone resistance in *Enterobacteriaceae*

E. coli 5' ATG GTG ACT CGG CGG TTT ATG ACA C 3'
(SEQ ID NO:25)
OR 5' ATG GTG ACT CGG CGG TCT ATG ACA C 3'
(SEQ ID NO26)

C. freundii 5' ATG GTG ATA CCG CCG TTT ACG ACA C 3'
(SEQ ID NO:27)

E. aerogenes 5' ATG GTG ATA CCG CCG TTT ATG ACA C 3'
(SEQ ID NO:28)

E. cloacae 5' ATG GTG ATT CCG CGG TGT ACG ACA C 3'
(SEQ ID NO:29)

K. oxytoca 5' ATG GTG ATA CTG CCG TAT ACG ACA C 3'
(SEQ ID NO:30)

K. pneumoniae 5' ACG GCG ACT CCG CGG TAT ACG ACA C 3'
(SEQ ID NO:31)

P. stuartii 5' ATG GTG ATA GCG CTG TTT ATG AGA C 3'
(SEQ ID NO:32)

S. marcescens 5' ACG GTG ACA GCG CGG TTT ACG ACA C 3'
(SEQ ID NO:33)